약학석사 학위논문

Potential Role of Leucyl-tRNA Synthetase in TGFβ1-Induced Epithelial-to-Mesenchymal Transition

TGFβ1에 의해 유도되는 EMT에서 LRS의 잠재적 역할에 대한 연구

2014 년 8 월

서울대학교 융합과학기술대학원

분자의학 및 바이오제약학과 의약생명과학전공

조 성 민

Potential Role of Leucyl-tRNA Synthetase in TGFβ1-Induced Epithelial-to-Mesenchymal Transition

TGFβ1에 의해 유도되는 EMT에서 LRS의 잠재적 역할에 대한 연구

지도교수 김 성 훈
이 논문을 약학석사 학위논문으로 제출함

2014 년 8 월

서울대학교 융합과학기술대학원
분자의학 및 바이오제약학과 의약생명과학 전공
조 성 민

조성민의 약학석사 학위논문을 인준함
2014 년 8 월

위 원 장 _____ (인)

부 위 원 장 _____ (인)

위 원 _____ (인)

Abstract

Recently, we reported that Leucyl-tRNA synthetase (LRS) has a non-canonical activity that regulates amino acid-induced mTORC1 activation. However, its specific functions in pathophysiological conditions have not been elucidated. To further our knowledge of the novel functions of LRS, we first obtained global LRS interactome data by affinity purification coupled to mass spectrometry (AP-MS) analysis. As a result, a total of 519 confident LRS interaction proteins were identified from the combination method of Strep-tag/Streptactin column-based LRS interactome purification and LC-MS/MS analysis (FDR ≤1%). we then employed a stringent filter with significance analysis of interactome (SAINT) and finally obtained 276 core interaction proteins with confidence score of ≥ 0.9. Through the gene ontology (GO) analysis, we found that LRS network harbors TGFβR-Smad axis as well as others such as TNF-NFkB signaling pathway. In addition, from MS based differentially expressed proteome (DEP) analysis of LRS wild type and heterozygous mouse embryonic fibroblast (MEF) cells, we observed that the expression levels of several types of collagens, which are known as the products of TGFβ signaling, were significantly increased in LRS heterozygous MEF cells. On this basis, we experimentally observed that forced LRS expression negatively regulates TGFβ1 induced migration rate of lung adenocarcinoma

cells via suppressing the C-terminal phosphorylation levels of Smad2/3 and the expression level of Snail, which represent the major transcription factors of epithelial cell-to-mesenchymal cell transition (EMT). Furthermore, we found that E3 ligase Nedd4L expression was significantly down-regulated in LRS heterozygous MEF cells and its positive correlation with LRS was also found in the late stage of lung cancer patients through tissue microarray immunohistochemical analysis. Collectively, from the newly obtained LRS global interactome and MEF derived DEP data, we found that LRS might have an important function in TGFβ1-associated cancer metastasis.

Keywords: LRS, AP-MS, SAINT analysis, TGFβ1 pathway, NEDD4L

Student ID: 2012-24153

Contents

Abstract ----------I

Contents ---------- III

List of figures and tables ----------IV

Introduction ----------1

Abbreviations list ----------4

Material and methods ----------5

Results ---------- 10

Discussion ---------- 23

References ----------27

국문초록 ---------- 32

List of figures and tables

Figure 1. Generation of LRS interactome by AP-MS approach and SAINT analysis ---------- 15

Figure 2. LRS plays a negative role in TGFβ1 induced EMT ---------- 16

Figure 3. LRS negatively regulates cell migration in A549 cells ---------- 17

Figure 4. Ubiquitin E3 ligase Nedd4l is down-regulated in LRS heterozygous MEF cell ---------- 18

Figure 5. Expression of LRS is positively correlated with that of TGFβsignaling ---------- 19

Table 1. Gene lists collected from LRS-TGFβpathway ---------- 20

Table 2. Expression levels of several types of collagens were increased in LRS heterozygous MEF cells. ---------- 21

Table 3. Relationship between LRS expression and Nedd4L in lung adenocarcinoma ---------- 22

Introduction

Aminoacyl-tRNAsynthetases (ARSs) are commonly known for catalyzing the ligation of amino acids to their cognate tRNAs. Especially, nine of twenty tRNA synthetases are assembled with three non-synthetase scaffold proteins, forming multi-tRNA synthetase complex (MSC).[1] Since ARSs are housekeeping enzymes, their non canonical roles beside protein synthesis were not expected. However, evidence on ARSs' non-canonical functions is mounting and their involvement in diseases such as cancer, angiogenesis, and inflammation has been discovered. [2,3]

Leucyl-tRNAsynthetase (LRS), ligating the leucine to $tRNA^{leu}$, is one of the MSC component and interacts with other components by its C-terminal domain.[4] Recently, its non-canonical function as a leucine sensor was reported; LRS activates mTORC1 signaling by facilitating GTP hydrolysis of RAGD in the presence of amino acids.[5] Also, interaction network of LRS was previously established based on 11 public databases.[3] However, since these gene lists were derived from different time, experiments, and numerous groups, the probability of their physical interactions in same context would be very low. To overcome this limitation, we aimed to draw highly accurate LRS interactome network by applying AP-MS approach and SAINT analysis.

Affinity purification coupled to mass spectrometry has been used widely to elucidate the perturbed dynamic interactome network of interesting target proteins in mammalian cells.[6-8] This approach provides valuable information of interconnectivity among proteins and discloses their potential biological functions. However, AP-MS data often contain non-specific binding proteins, and therefore the filtering of these false positive proteins has become an issue in the proteomics field.[9] To overcome such challenge, we employed a recently developed SAINT algorithm, which uses spectral counting to compute the confidence scores of protein-protein interactions.[10,11] As SAINT analysis discerns non-specific binding proteins by utilizing the negative control data, we could generate highly confident interactome data for network interpretation.

Transforming growth factor β (TGF-β) has been known to control cell phenotypes by regulating expression of key determinant genes involved in cell differentiation, cell cycle, cell adhesion, and etc.[12,13] Therefore, the study of mechanism of TGFβ signal transduction has been performed intensively for past several years. TGFβ signaling is activated starting with TGFβ family ligands such as TGFβs, Activin or Nodal binds to type 2 receptor and recruit type1 receptor to form hetero-tetramermic complex.[14] When the serine residue of TGFβR1 is phosphorylated by type2 receptor, TGFβR1 can transmit signal through phosphorylating the serine

residue of the Smad2/3(c-terminal phosphorylation).[15] Then, Smad2/3 forms a complex with Smad4 with high affinity and this complex can enter the nucleus where it can act as a transcription factor[16].

In this study, AP-MS approach coupled to SAINT analysis allow us to discern false positive proteins, thereby collecting highly confident LRS interactome data. Using this LRS dataset, we expect that LRS might have a novel function in TGFβ pathway. Throughout the experimental validations, we showed that LRS negatively regulates TGFβ pathway which suggests that LRS mediated Neural Precursor Cell Expressed, Developmentally Down-Regulated 4-Like, E3 ubiquitin protein ligase (Nedd4L) regulation may be the cause.

Abbreviations list

LRS: Leucyl-tRNA synthetase

MSC: Multi-tRNA synthetase complex

EV: Empty vector

WCL: Whole cell lysate

AP-MS: Affinity purification-Mass spectrometry

SAINT: Significance analysis of interactome

MEF: Mouse embryonic fibroblast

Material and methods

Cell culture and materials

HEK293T cells were grown in DMEM and A549 cells were maintained in RPMI medium containing 10% fetal bovine serum with 1% antibiotics at 37℃ in a 5% CO_2 incubator. N-terminal and C-terminal strep-tagged LRS were used with Fugene HD and Lipofectamine 2000 transfection reagents for DNA transfection, following the manufacturer's instruction. Cells were fractionated into cytoplasm and nucleus by nuclear extract kit (Active motif) and TGFβ1 (10 ng/ml) was purchased from R&D systems.

Cell migration assay

Cell migration assays with a549 cells were performed in transwell chambers (8.0 μM pore, costar) as described.[17] Fibronectin (10ug/ml,BD Biosciences) was coated on the membrane and 700ul(serum free) was placed in the bottom chamber. After a549 cells were trypsinized and centrifuged at 1,000 rpm for 5 minutes, serum-free RPMI was added to the cells for suspension. Then, 1×10^5 cells were dropped into 24-well Transwell chambers and incubated for 12 h at 37℃ in a CO_2 incubator. After washing the membrane once with PBS, 70% methyl alcohol in PBS was used for cell fixation. PBS washing was performed three times afterwards and

hematoxylin(Sigma) was used for cell staining. After washing the membrane with distilled water a couple of times, non-migrant cells from the top were removed by using cotton swab. Then, the membranes were mounted with Gel Mount (Biomeda, Foster City, CA). For counting migrant cells, three randomly picked places were magnified with microscopes in high-power fields (×10, and ×20). All samples were performed in triplicate.

Western Blot analysis

After A549 cells were lysed with lysis buffer (0.5% triton-X100, 25 mM tris, 0.5 mM EDTA, 10 mM NaCl, protease inhibitor) and incubated in 4 ℃ for 30 minutes, lysates were collected in a 1.5 ml tube and centrifuged at 13,500 rpm for 10 minutes. Then, supernatants were collected and quantified by using Bradford reagent. After 5× sample buffer was added, each sample were boiled in 100℃ for 5 minutes. 20-30 μg of proteins from each sample were then loaded to 10% acrylamide gels. Then, proteins were transferred onto a PVDF membrane for 40 minutes. After letting the primary antibody attached to the membrane overnight, membrane was then washed 3times in TBS-T for 5minutes each. Then, secondary antibodies (1:5,000) were attached to the membrane and TBS-T washing step were performed 3 times, 5minutes each. Finally, bands were visualized by using the chemiluminescent reagent.

Determination of LRS and Nedd4L expression levels in lung adenocarcinoma tissue microarray samples

Lung adenocarcinoma tissue microarrays (LC1504) were purchased from Biomax. Tissues were incubated in xylene for 5 minutes, for 3 times. Then immersed in ethanol twice for 5minutes and then 90, 80, 70% ethanol for 5 minutes in a sequential manner. Finally, tissues were washed with distilled water (DW). Next, tissues were treated with 0.3# hydrogen peroxide for 15 minutes and washed with DW. The antigens in the tissues were retrieved from the tissues in citrate buffer (pH 6.0) using microwave and washed with DW and then PBS. Then, tissues were blocked with PBS-T containing 4% BSA and washed with PBS-T. After tissues were incubated with anti-LRS (1:300 dilution) and Nedd4L (1:300 dilution) antibodies for 1h at RT, tissues were washed with PBS-T for 5 minutes, 3 times each. Then, horseradish peroxidase (HRP)-labeled polymer anti-rabbit secondary antibody was incubated for 1h at RT, washed for 5min three times, and finally by PBS-T for 5 min three times. Then, 1 ml of DAB substrate buffer was treated and chromogen 20 µl and washed with tap water. The tissues were stained with Mayer's hematoxylin and then washed. After dehydration, the staining intensity of LRS and Nedd4L was graded into three different levels.

LC-MS/MS based LRS interactome profiling and SAINT analysis

After ectopic expression of C-terminal or N-terminal Twin-strep tagged LRS or mocks in HEK 293 T cells, purified LRS interactome, from streptactin column system were separated with 1D-SDS-PAGE. Then, tryptic peptides were eventually purified from In-Gel trypsin digestion process. LC-MS/MS analysis of each tryptic peptides was subsequently performed. Each peptides were separated by online reversed-phase chromatography for each run using a Thermo Scientific Eazy nano LC II autosampler with a reversed-phase peptide trap EASY-Column (100 μm inner diameter, 2 cm length) and a reversed-phase analytical EASY-Column (75 μm inner diameter, 10 cm length, 3 μm particle size, both Thermo Scientific), and electrospray ionization was subsequently performed using a 30 μm (i.d.) nano-bore stainless steel online emitter (Thermo Scientific) and a voltage set at 2.6 V., at a flow rate of 300 nl/min. protein identification was accomplished utilizing the Proteome Discoverer v1.3 database search engine (Thermo scientific) and searches were performed against IPI. Human.v3.87 database. The carbamidomethylation (+57.021 Da) of cystein (C) or deamidated (+0.984 Da) of asparagine or glutamine (N, Q) is set as a static modification or as a variable modification, respectively.

Differentially expressed proteome analysis of LRS heterozygous mouse embryonic fibroblast (MEF) cells

MEFs were derived from wild type and LRS heterozygous littermates.

MEF cells were maintained in DMEM medium containing 10% fetal bovine serum with 1% antibiotics at 37℃ in a 5% CO_2 incubator. Cells from both types were lysed in RIPA lysis buffer containing protease inhibitors. Tryptic peptides produced from In Solution digestion and were analyzed by LC-MS/MS analysis. Subsequent procedure is the same as described above.

Results

Generation of LRS interactome by AP-MS approach and SAINT analysis

To identify the novel interaction partners of LRS, we fused N- or C- terminal twin-strep tag to LRS and transiently transfected into HEK293T cells, respectively. For the negative controls, N-or C-terminal twin-strep tag alone vectors were used. Purified LRS interaction proteins from strep-tactin column system were subsequently identified by a LC-MS/MS analysis. (peptide probability≥ 95%, protein probability ≥ 95%, unique peptide ≥2), we identified a total of 516 LRS interaction partners. Next, to remove non specific binding proteins and find true interaction partners, these initially filtered 516 LRS interaction proteins were subjected to SAINT analysis for the second step of filtration. We compared the spectral count distribution between LRS and negative control to remove false positive interaction partners through SAINT analysis and obtained a total of 276 interaction proteins (Figure 1).

Gene Ontology (GO) analysis of LRS Interactome

In order to explore the LRS interactome network, we used the Netvenn analysis programme, which interprets gene relationships

and potential biological function from huge datasets (http://wheat.pw.usda.gov/NetVenn/)[18]. The analyzed results indicates that LRS interactome network had a strong connection to TGFβR signaling (p-value < 9.7×10^{-5}) as well as TNF/NF-kB signaling pathway (p-value < 1.7×10^{-10}). Out of 276 LRS interaction proteins, 7 of them were selected (Table1) as the gene lists that might have an association with TGFβR-Smad pathway. To find the potential function of LRS, we examined these selected proteins and found that NUP153, NUP214, KPNB1, and HSPA8 were involved (Table 1) in Smad transporting mechanism.[19-22] Since Smads are considered as major regulators of TGFβ signaling pathway, we predicted that LRS may have an unknown function in TGFβ signaling through the regulation of Smads or its related molecules.

Expression levels of various collagen types are increased in LRS heterozygous MEF cells

To gain further insight into the biological functions of LRS, we carried out differentially expressed proteins analysis from LRS heterozygous mouse embryonic fibroblast (MEF) cells and its wild type. Purified proteins from both cell lines were analyzed by LC-MS/MS analysis after in solution digestion. Interestingly, we found an increase in various collagen types in LRS hetero MEF cells (Table 2). Since TGFβstimulates the collagen synthesis,[23-25] we predicted that

the loss of LRS may contribute to TGFβsignaling regulation. Combined with the results from Netvenn analysis, we expected that LRS may have a non-canonical function in TGFβpathway.

LRS plays a negative role in TGFβ1 induced EMT

From LRS interactome functional GO analysis, we found that CDK4, and EIF3I have known to be involved (Table 1) in TGFβ1 induced cancer epithelial cell-to-mesenchymal cell transition (EMT) process.[26,27] To investigate the possibility of a novel role of LRS in TGFβ linked cancer metastasis, we first checked expression level of LRS in both cytoplasm and nucleus during TGFβ1 induced EMT process of A549 lung adenocarcinoma cells. Interestingly, expression of LRS decreased continuously by TGFβ1 in a time-dependent manner (Figure 2A). Therefore, we predicted that LRS may play a negative role in TGFβ1 pathway. To check the effects of LRS in TGFβ1 pathway, LRS was over-expressed in A549 cells and treated with TGFβ1 or not. Major transcription factors involved in TGFβ1 induced EMT process such as p-Smad2, 3 and Snail were significantly decreased (Figure 2B).[15,16,28] Expression level of Fibronectin, known as a mesenchymal marker[29], also decreased in later stage of TGFβ1 EMT process (Figure 2C). Next, we checked the functional role of LRS in TGFβ1 induced migration of A549 cells since cancer cells that undergo EMT process acquire migratory

character,[30] and this property was checked in LRS over-expressed cells. Using trans-well coated with fibronectin for migration assay, we found that migration ability was decreased by LRS over-expression (Figure 3). Overall, these results suggest LRS is negatively involved in transcriptional regulation and control EMT markers in early and later stages of TGFβ1 induced EMT process.

Ubiquitin E3 ligase Nedd4L is down-regulated in LRS heterozygous MEF cell

To gain a better understanding of how TGFβ1 pathway is regulated by LRS, we predicted that E3 ligase might be involved in this process. According to recent studies, E3 ligase Nedd4L was reported as the major mediator of degrading TGFβ1 activated Smads.[31,32] Since Nedd4L tightly regulate TGFβ1 dependent phosphorylation of Smads, we checked its level in LRS heterozygous MEF cells. Surprisingly, we found that E3 ligase Nedd4L expression is significantly down-regulated in LRS heterozygous MEF cells, suggesting that LRS might control TGFβ1 pathway through Nedd4L regulation (Figure 4). Although, phosphorylated Smad2 was up-regulated in wild type MEF cells with a TGFβ1 treatment of 30 minutes, its level decreased dramatically later on. Since the active form of Smad2 is prolonged in LRS heterozygous MEF cells, we reckoned that LRS mediated Nedd4L down-regulation augments the

accumulation of active form of Smads.

Expression of LRS is positively correlated with that of Nedd4L

To examine whether the relationship between LRS and Nedd4L present in tumor tissues, the expression of LRS and Nedd4L was compared in human lung adenocarcima by immunostaining(Figure 5). Expression pattern of LRS and Nedd4L was analyzed from 52 cases of grade 2 lung adenocarcinoma and 41 cases of grade 3 lung adenocarcinoma. The expression level of LRS is positively correlated with that of Nedd4L (Table 3) and these observations indicate that LRS-Nedd4L-Smads axis may be involved in the lung cancer metastasis.

Figure 1. Generation of LRS interactome by AP-MS approach and SAINT analysis

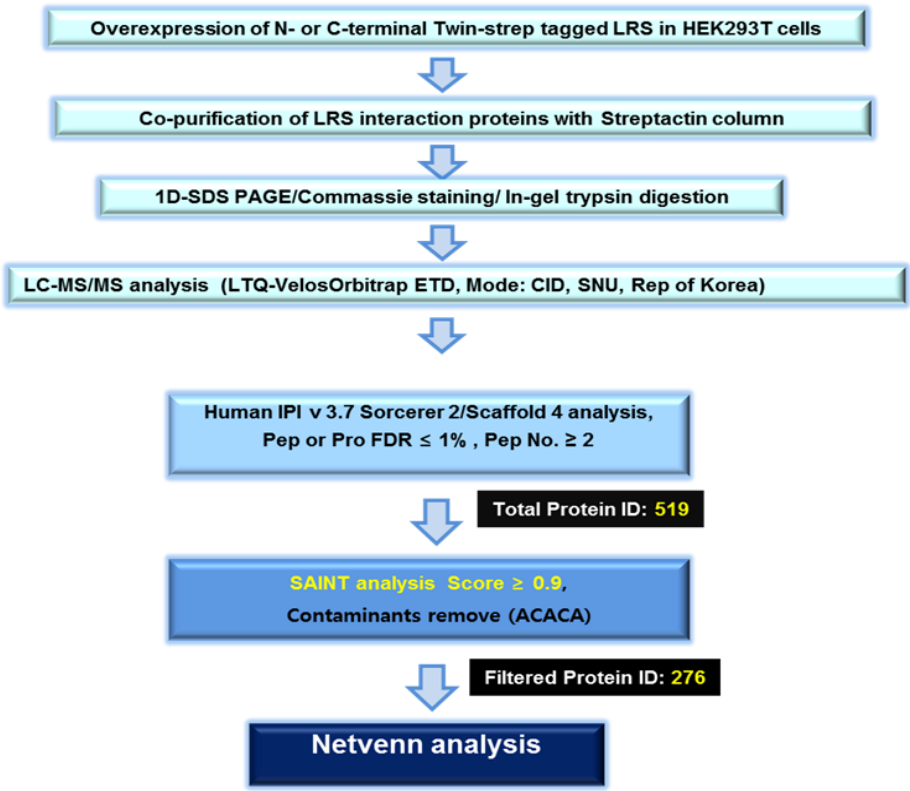

Figure 1. Generation of LRS interactome by AP-MS approach and SAINT analysis. LRS clone tagged with n or c terminus twin-strep was transfected in HEK293T cells and subjected to affinity purification coupled to mass spectrometry. Using human database, we identified 519 proteins in total. After that, using SAINT analysis with a confidence score higher than 0.9, we obtained 276 interaction proteins. Finally we used Netvenn analysis for further applications.

Figure 2. LRS plays a negative role in TGF β 1 induced EMT

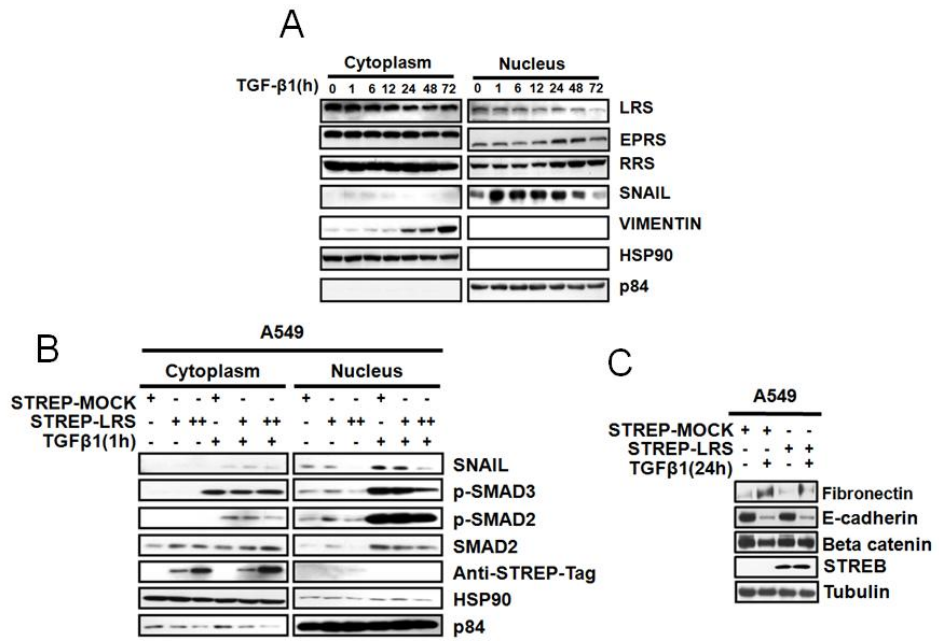

Figure 2. LRS plays a negative role in TGFβ1 induced EMT. A) A549 cells were treated with TGFβ1 for indicated times, fractionated into cytoplasm and nucleus, and then immunoblotting was performed using an anti-LRS antibody. B) Cells were transfected with strep-LRS or EV for 24 h and then stimulated with TGFβ1 (10 ng/ml) for 1 h. Then, the expression of Smad pathway molecules and snail were detected by immunoblotting after cells were fractionated into cytoplasm and nucleus. Hsp90 and p84 were used as a loading control for the cytoplasmic and nucleus fraction respectively. C) Cells were transfected with strep-LRS or EV for 24 h and then stimulated with TGFβ1 (10 ng/ml) for 24 h. E-cadherin and Fibronection antibodies were used for immunoblotting.

Figure 3. LRS negatively regulates cell migration in A549 cells

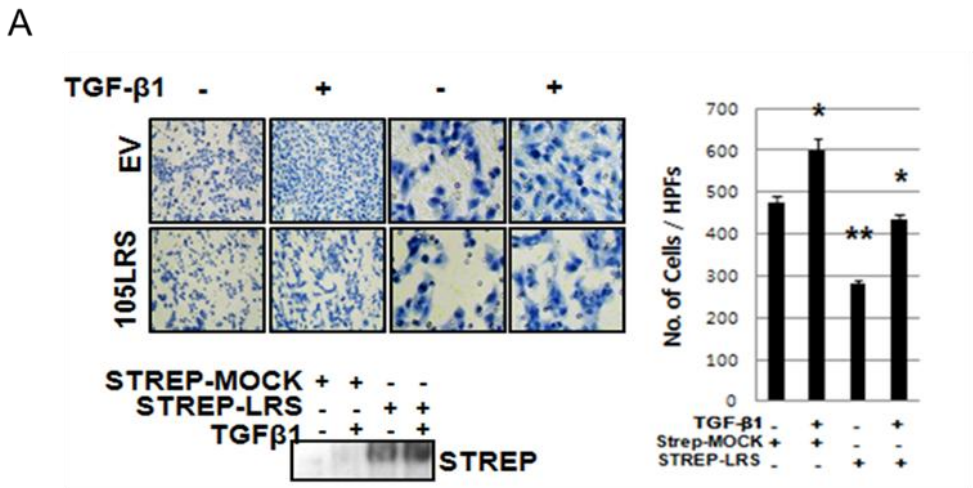

Figure 3. LRS negatively regulates cell migration in A549 cells. A) Over-expression of LRS regulates migration phenotype of A549 cells. A549 cells were transfected with Strep-LRS or EV and subjected to migration assay in the absence or presence of TGFβ1. Migrated cells through the membrane were counted. Three independent experiments were plotted.

Figure 4. Ubiquitin E3 ligase Nedd4l is down-regulated in LRS heterozygous MEF cell

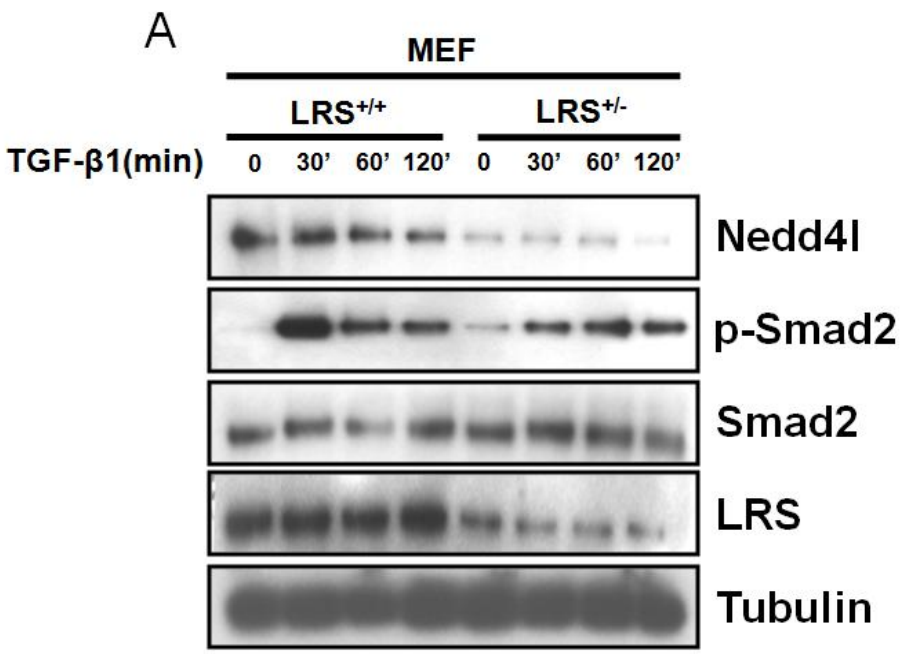

Figure 4. Ubiquitin E3 ligase Nedd4l is down-regulated in LRS heterozygous MEF cell A) $LRS^{+/+}$ and $LRS^{+/-}$ Mouse embryonic fibroblast (MEF) cells were treated with TGFβ1 or indicated times. Extracts from the cells were subjected to immunoblotting.

Figure 5. Expression of LRS is positively correlated with that of TGF β signaling

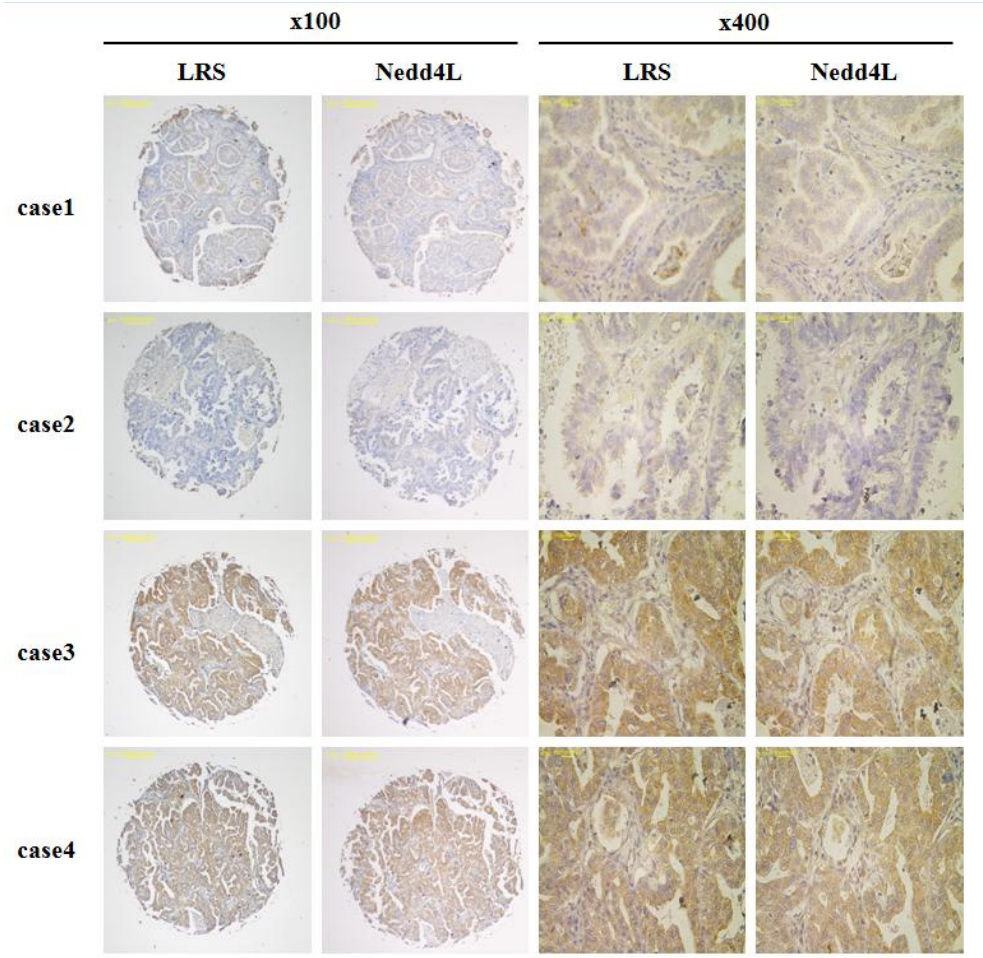

Figure 5. Expression of LRS is positively correlated with that of Nedd4L. Expression pattern of LRS and Nedd4L was analyzed from 52 cases of grade2 lung adenocarcinoma and 41 cases of grade 3 lung adenocarcinoma. The staining intensity of LRS and Nedd4L was graded into three different levels. The expression of LRS is positively correlated with that of Nedd4L.

Table 1. Gene lists collected from LRS-TGF β pathway

Gene	Description	Location	Family
CDK1	cyclin-dependent kinase 1	Nucleus	kinase
CDK4	cyclin-dependent kinase 4	Nucleus	kinase
HSPA8	heat shock 70kDa protein 8	Cytoplasm	enzyme
KPNB1	karyopherin (importin) beta 1	Nucleus	transporter
EIF3I/ TRIP1	eukaryotic translation initiation factor 3, subunit I (TGFbeta Receptor-Interacting Protein 1)	Cytoplasm	translation regulator
NUP153	nucleoporin 153kDa	Nucleus	transporter
NUP214	nucleoporin 214kDa	Nucleus	transporter

Table1. Gene lists collected from LRS-TGFβ pathway. Out of 276 LRS interaction proteins, 7 of them were selected (Table1) as the gene lists that might have an association with TGFβR-Smad pathway

Table 2. Expression levels of several types of collagens were increased in LRS heterozygous MEF cells.

Description	IPI number	Fold change
Collagen, type V, alpha 1	IPI00128689	14.29
Collagen, type VI, alpha 3	IPI00877197	10.00
Collagen alpha-1(V) chain	IPI00128689	5.00
Collagen alpha-1(VI) chain	IPI00339885	3.33
Isoform 2 of Collagen alpha-1(I) chain	IPI00623191	1.67
Collagen alpha-2(V) chain	IPI00121120	1.67
Collagen, type III, alpha 1	IPI00129571	1.43
Isoform 1 of Collagen alpha-1(XII) chain	IPI00121430	1.25
Collagen, type I, alpha 1	IPI00329872	1.11

Table 2. Expression levels of several types of collagens were increased in LRS heterozygous MEF cells. We checked differentially expressed proteins that might have a relationship with TGFβ1 pathway. Interestingly, we found an increase in various collagen types in LRS hetero MEF cells

Table 3. Relationship between LRS expression and Nedd4L in lung adenocarcinoma

		LRS			
		Negative	Low	High	P-value
Nedd4L	Negative	34	4	3	
	Low	9	11	9	
	High	8	3	12	
					< 0.0001

Table 3. Relationship between LRS expression and Nedd4L in lung adenocarcinoma. Expression pattern of LRS and Nedd4L was analyzed from 52 cases of grade2 lung adenocarcinoma, and 41 cases of grade 3 lung adenocarcinoma. The staining intensity of LRS and Nedd4L was graded into three different levels. Correlation between LRS and Nedd4L was analyzed by fisher's exact test.

Discussion

The multiple steps of carcinogenesis with the accumulation of genetic mutations have been studied extensively for several decades and cancer treatments such as chemotherapy, surgical resection and radiotherapy have been developed based on these studies.[33] However, responses to therapies are often short-lived as tumor cells acquire drug resistance.[34] As a result, pan-resistance state raised the rate of distant recurrence (metastasis) and this process is involved in the majority of cancer deaths.[35] Recently, several studies report that EMT is a required step for metastasis and closely related to drug resistance.[36,37] Since EMT confers migratory and invasive characters that lead to cancer dissemination, suppressing or reversing the process of EMT would be a breakthrough for the metastasis therapy. In this study, we found that LRS has a negative role in TGFβ1 induced EMT, implying that further study on LRS would provide novel therapeutic targets.

Recently, the non-canonical function of LRS in amino acid-induced mTORC1 activation was reported. Since mTORC1 was reported to regulate TGFβ1 induced EMT process, we initially expected the function of LRS as a positive regulator of this process.[38] However, we observed that LRS has a negative role in TGFβ1 induced EMT process in our experiments. we showed that over-expression of LRS attenuated TGFβ1 induced EMT through

suppressing the expression level of Snail and active form of Smad2/3. Moreover, forced LRS expression suppressed the cell migration rate which is the key phenotype of EMT process. With the given results, we suggest that LRS has another non-canonical function beside the mTORC1 activation.

NEDD4L is a critical E3 ligase in TGFβ1 pathway and its clinical importance is well reported. [39-42] During TGFβ1 stimulation, TGFβ1 can induce Smad2/3 linker phosphorylation mediated by CDK8, and CDK9 for proteasomal destruction.[43] Then, E3 ligase Nedd4L can recognize the phosphorylated linker sites and lead the Smad 2/3 polyubiquitination process.[32] Since Nedd4L suppresses TGFβ1 responsiveness by regulating the activated form of Smad2/3 turnover, the control of Nedd4L expression in TGFβ1 related diseases would be a potential therapeutic target.[31] Throughout the study, we showed that Nedd4L level is down-regulated in LRS heterozyogous MEF cells. Also, the active form of Smad2 is prolonged in LRS heterozygous MEF cells, suggesting that the consequence is due to the loss of Nedd4L. We also found the correlation between LRS and Nedd4L in lung adenocarcinoma tissue array. The result from tumor tissue immunostaining supports the idea that Nedd4L might be regulated by LRS. Thus, the study of the mechanism on Nedd4L regulation by LRS could present a pontential therapeutic opportunity.

Previously, interaction partners of LRS were collected from 11 public databases and the association of LRS with cancer associated genes was once reported.[3] However, public databases are often generated from different experimental conditions; thereby the generation of reliable information with biological significance was often considered as a challenging work. To overcome this issue, we carried out AP-MS approach with SAINT analysis to obtain highly accurate interactome network.

Netvenn analysis of the interactions network mediated by LRS allows us to expect its novel function in TGFβ1 pathway. However, we also found that LRS interactome network had a strong connection to TNF-α signaling. Although, an in depth study on LRS mediated TNF-α signaling is needed, we suggest that LRS might have a potential importance in cancer by regulating TGFβ1 and TNF-α signaling pathways.

In conclusion, we generated LRS interactome with AP-MS and SAINT analysis to discover the novel function of LRS. As a result, a total of 276 proteins were identified and we found that the LRS interactome network is potentially related with the TGFβ1 pathway. Throughout the study in investigating the role of LRS in TGFβ1pathway, we could conclude that LRS attenuates TGFβ1 induced EMT process through Smad signaling pathway and this process might be mediated by Nedd4L regulation. Overall, we

showed that AP-MS with saint analysis is a great strategy to find accurate interactome and has a potential to have a broad impact on biology and medicine.

References

1. Park, S.G., Ewalt, K.L. & Kim, S. Functional expansion of aminoacyl-tRNA synthetases and their interacting factors: new perspectives on housekeepers. *Trends in biochemical sciences* **30**, 569-574 (2005).
2. Guo, M. & Schimmel, P. Essential nontranslational functions of tRNA synthetases. *Nature chemical biology* **9**, 145-153 (2013).
3. Kim, S., You, S. & Hwang, D. Aminoacyl-tRNA synthetases and tumorigenesis: more than housekeeping. *Nature reviews. Cancer* **11**, 708-718 (2011).
4. Ling, C., *et al.* The C-terminal appended domain of human cytosolic leucyl-tRNA synthetase is indispensable in its interaction with arginyl-tRNA synthetase in the multi-tRNA synthetase complex. *The Journal of biological chemistry* **280**, 34755-34763 (2005).
5. Han, J.M., *et al.* Leucyl-tRNA synthetase is an intracellular leucine sensor for the mTORC1-signaling pathway. *Cell* **149**, 410-424 (2012).
6. Pardo, M. & Choudhary, J.S. Assignment of protein interactions from affinity purification/mass spectrometry data. *Journal of proteome research* **11**, 1462-1474 (2012).
7. Plocinski, P., *et al.* Identification of protein partners in mycobacteria using a single-step affinity purification method. *PloS one* **9**, e91380 (2014).
8. Moon, S., *et al.* Interactome analysis of AMP-activated protein kinase (AMPK)-alpha1 and -beta1 in INS-1 pancreatic beta-cells by affinity purification-mass spectrometry. *Scientific reports* **4**, 4376 (2014).
9. Nesvizhskii, A.I., Vitek, O. & Aebersold, R. Analysis and validation of proteomic data generated by tandem mass spectrometry. *Nature methods* **4**, 787-797 (2007).
10. Choi, H., *et al.* SAINT: probabilistic scoring of affinity

purification-mass spectrometry data. *Nature methods* **8**, 70-73 (2011).

11. Choi, H., Glatter, T., Gstaiger, M. & Nesvizhskii, A.I. SAINT-MS1: protein-protein interaction scoring using label-free intensity data in affinity purification-mass spectrometry experiments. *Journal of proteome research* **11**, 2619-2624 (2012).
12. Siegel, P.M. & Massague, J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nature reviews. Cancer* **3**, 807-821 (2003).
13. Massague, J. TGF-beta signaling in development and disease. *FEBS letters* **586**, 1833 (2012).
14. Lin, H.Y. & Moustakas, A. TGF-beta receptors: structure and function. *Cellular and molecular biology* **40**, 337-349 (1994).
15. Shi, Y. & Massague, J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700 (2003).
16. Massague, J. & Wotton, D. Transcriptional control by the TGF-beta/Smad signaling system. *The EMBO journal* **19**, 1745-1754 (2000).
17. Park, S.G., *et al.* Human lysyl-tRNA synthetase is secreted to trigger proinflammatory response. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 6356-6361 (2005).
18. Wang, Y., Thilmony, R. & Gu, Y.Q. NetVenn: an integrated network analysis web platform for gene lists. *Nucleic acids research* (2014).
19. Yahata, T., *et al.* The MSG1 non-DNA-binding transactivator binds to the p300/CBP coactivators, enhancing their functional link to the Smad transcription factors. *The Journal of biological chemistry* **275**, 8825-8834 (2000).
20. Kurisaki, A., Kose, S., Yoneda, Y., Heldin, C.H. & Moustakas, A. Transforming growth factor-beta induces nuclear import of Smad3 in an importin-beta1 and Ran-dependent manner. *Molecular biology of the cell* **12**, 1079-1091 (2001).
21. Xiao, Z., Liu, X. & Lodish, H.F. Importin beta mediates nuclear

translocation of Smad 3. *The Journal of biological chemistry* **275**, 23425-23428 (2000).

22. Xu, L., Kang, Y., Col, S. & Massague, J. Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFbeta signaling complexes in the cytoplasm and nucleus. *Molecular cell* **10**, 271-282 (2002).
23. Varga, J., Rosenbloom, J. & Jimenez, S.A. Transforming growth factor beta (TGF beta) causes a persistent increase in steady-state amounts of type I and type III collagen and fibronectin mRNAs in normal human dermal fibroblasts. *The Biochemical journal* **247**, 597-604 (1987).
24. Shao, J., Xia, Z.W., Li, Y.Z., Yu, S.C. & Deng, W.W. [Expression of cellular phenotype switching markers-matrix protein Gla, mRNA and collagen I, III and V of human airway smooth muscle cells in vitro after TGF-beta1 stimulation]. *Zhonghua er ke za zhi. Chinese journal of pediatrics* **44**, 531-534 (2006).
25. Chen, S.J., *et al.* Stimulation of type I collagen transcription in human skin fibroblasts by TGF-beta: involvement of Smad 3. *The Journal of investigative dermatology* **112**, 49-57 (1999).
26. Perez, R.E., Navarro, A., Rezaiekhaligh, M.H., Mabry, S.M. & Ekekezie, II. TRIP-1 regulates TGF-beta1-induced epithelial-mesenchymal transition of human lung epithelial cell line A549. *American journal of physiology. Lung cellular and molecular physiology* **300**, L799-807 (2011).
27. Liu, F. & Korc, M. Cdk4/6 inhibition induces epithelial-mesenchymal transition and enhances invasiveness in pancreatic cancer cells. *Molecular cancer therapeutics* **11**, 2138-2148 (2012).
28. Wang, Y., Shi, J., Chai, K., Ying, X. & Zhou, B.P. The Role of Snail in EMT and Tumorigenesis. *Current cancer drug targets* **13**, 963-972 (2013).
29. Klein-Scory, S., *et al.* High-level inducible Smad4-reexpression in the cervical cancer cell line C4-II is associated with a gene expression profile that predicts a

preferential role of Smad4 in extracellular matrix composition. *BMC cancer* **7**, 209 (2007).

30. Samatov, T.R., Tonevitsky, A.G. & Schumacher, U. Epithelial-mesenchymal transition: focus on metastatic cascade, alternative splicing, non-coding RNAs and modulating compounds. *Molecular cancer* **12**, 107 (2013).
31. Gao, S., *et al.* Ubiquitin ligase Nedd4L targets activated Smad2/3 to limit TGF-beta signaling. *Molecular cell* **36**, 457-468 (2009).
32. Kuratomi, G., *et al.* NEDD4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) negatively regulates TGF-beta (transforming growth factor-beta) signalling by inducing ubiquitin-mediated degradation of Smad2 and TGF-beta type I receptor. *The Biochemical journal* **386**, 461-470 (2005).
33. Schmitt, M.W., Prindle, M.J. & Loeb, L.A. Implications of genetic heterogeneity in cancer. *Annals of the New York Academy of Sciences* **1267**, 110-116 (2012).
34. Maemondo, M., *et al.* Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *The New England journal of medicine* **362**, 2380-2388 (2010).
35. Jones, S.E. Metastatic breast cancer: the treatment challenge. *Clinical breast cancer* **8**, 224-233 (2008).
36. Nurwidya, F., Takahashi, F., Murakami, A. & Takahashi, K. Epithelial mesenchymal transition in drug resistance and metastasis of lung cancer. *Cancer research and treatment : official journal of Korean Cancer Association* **44**, 151-156 (2012).
37. Zhang, L., Wu, Z. & Zhou, Q. [Epithelial-mesenchymal transition and tumor drug resistance]. *Zhongguo fei ai za zhi = Chinese journal of lung cancer* **16**, 54-57 (2013).
38. Gulhati, P., *et al.* mTORC1 and mTORC2 regulate EMT, motility, and metastasis of colorectal cancer via RhoA and Rac1 signaling pathways. *Cancer research* **71**, 3246-3256 (2011).

39. Hu, X.Y., Xu, Y.M., Fu, Q., Yu, J.J. & Huang, J. Nedd4L expression is downregulated in prostate cancer compared to benign prostatic hyperplasia. *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology* **35**, 527-531 (2009).
40. Gao, C., Pang, L., Ren, C. & Ma, T. Decreased expression of Nedd4L correlates with poor prognosis in gastric cancer patient. *Medical oncology* **29**, 1733-1738 (2012).
41. He, S., *et al.* Down-regulation of Nedd4L is associated with the aggressive progression and worse prognosis of malignant glioma. *Japanese journal of clinical oncology* **42**, 196-201 (2012).
42. Sakashita, H., *et al.* Identification of the NEDD4L gene as a prognostic marker by integrated microarray analysis of copy number and gene expression profiling in non-small cell lung cancer. *Annals of surgical oncology* **20 Suppl 3**, S590-598 (2013).
43. Alarcon, C., *et al.* Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-beta pathways. *Cell* **139**, 757-769 (2009).

국문초록

TGFβ1에 의해 유도되는 EMT에서 LRS의 잠재적 역할에 대한 연구

Aminoacyl-tRNA synthetases (ARSs)는 아미노산을 상보적인 tRNA에 ligation 을 촉진하여 단백질 합성에 필수적인 Aminoacyl-tRNA를 생성하는 효소로써, 11개의 ARS는 free standing form으로 나머지 9개의 ARS는 3개의 Aminoacyl-tRNA synthetase-interacting multifunctional proteins (AIMPs)과 더불어 multi-tRNA synthetase complex (MSC)를 형성한다. 최근에, MSC를 이루고 있는 각각의 ARS들은 단백질 번역뿐만 아니라, 다양한 환경에 의해 다이나믹하게 활성화되어 하부 신호전달 경로를 결정하는 핵심 조절자 역할을 수행하는, ‘ex-tranlastional activity’ 기능 또한 중요한 것으로 보고 되고 있다. 그 중, Leucyl-tRNA synthetase (LRS)는 glutamyl-prolyl-tRNA synthetase (EPRS) 및 isoleucyl-tRNA synthetase (IRS)와 함께 MSC 의 subdomain III 을 형성하며, leucine sensor로써 mTORC1 activation을 조절하는 non-canonical 역할이 알려져 있다. 본 연구에서는, LRS의 보고 되지 않은 결합단백체 (interaction partners) 정보를 통해 추가적인 기능 및 질환 관련성 여부를 확인하고자 Affinity purification-mass spectrometry (AP-MS) 기법과 Significance analysis of interactome (SAINT) 분석을 통한 LRS의 global interactome 분석을 수행하였다. N 혹은 C 말단 Twin-Strep tagged LRS를 HEK 293T에 과발현 및 단백체 분리 후 Streptactin 컬럼을 이용하여 LRS 결합단백체를

정제한 뒤에 LC-MS/MS 분석을 통해 519개의 LRS interaction proteins을 동정하였다. SAINT 분석을 통해 총 276개의 신뢰도 높은 LRS interaction proteins을 확보하였다 (Score ≥ 0.9). 그 후 Netvenn network 분석을 통해 CDK1, CDK4, HSPA8, KPNB1, EIF3I/TRIP1, NUP153, 혹은 NUP214 등이 TGFβ/Smad pathway 에 관련이 있다는 점을 확인하였다. 또한, LC-MS/MS 분석을 통해 LRS heterozygous 및 WT의 MEF cells의 단백체의 발현량을 비교분석 한 결과 heterozygous MEF에서 TGFβ에 의해 생산되는 것으로 알려진 다양한 타입의 collagens 발현이 증가함을 확인함으로써 LRS가 TGFβ pathway에 직접적으로 관련이 있을 수 있음을 예측할 수 있었다. 또 한, LRS의 과발현이 Smad 2/3의 C-terminal 인산화를 통해 TGFβ1의 신호전달을 억제시키며 Epithelial to Mesenchymal Transition (EMT) process에서 중요한 성질인 cell migration과 전사인자인 Snail 또한 억제함을 확인하였다. 마지막으로, E3 ligase인 Nedd4L이 LRS heterozygous MEF cell에서 감소되는 반면, TGFβ자극에 의한 Smad 2의 C-terminal 인산화가WT에 비해 더 지속됨을 확인하였고, lung adenocarcinoma 환자 유래의 tissue microarray (TMA)를 이용한 immnunohistochemistry 실험을 통해 약 36%(34/94)의 시료의 같은 부위에서 LRS 및 Nedd4L가 모두 유사하게 매우 낮은 발현률을 보이는 것을 관찰 하였다. 따라서, 본 연구를 통해 LRS 는 Nedd4L-Smad axis를 조절함으로써 암 전이에 직접적으로 관여 할 수 있으며, 새로운 약물 타깃이 될 수 있음을 밝혀내었다.

Keywords: LRS, AP-MS, SAINT analysis, TGFβ1 pathway, NEDD4L

주요어: Leucyl-tRNA synthetase (LRS), AP-MS, SAINT 분석, TGFβ1 신호, NEDD4L

학번: 2012-24153